

**REMARKS**

Reconsideration and withdrawal of the rejections of the May 17, 2006 Office Action is respectfully requested in view of the remarks and amendments herewith.

**I. STATUS OF THE CLAIMS AND FORMAL MATTERS**

Claims 1, 3-4, 9, 11-48 and 50-75 are now pending. Claims 1 and 4 have been amended, without prejudice, without admission, without surrender of subject matter, and without any intention of creating any estoppel as to equivalents.

No new matter is added.

It is submitted that these claims are and were in full compliance with the requirements of 35 U.S.C §112. In addition, the amendment and remarks herein are not made for the purpose of patentability within the meaning of 35 U.S.C. §§101, 102, 103 or 112; but rather the amendments and remarks herein are made simply for clarification and to round out the scope of protection to which Applicants are entitled. Support for the amended claims is found throughout the specification and the original claims.

**II. THE REJECTIONS UNDER 35 U.S.C. §112 ARE OVERCOME**

Claim 4 was rejected under 35 U.S.C. §112, second paragraph, as allegedly being indefinite for failing to point out and distinctly claim the subject matter of the invention. Applicants respectfully traverse.

The Office Action stated that the recitation “a linking polypeptide which binds r is attached” was unclear as it was not certain what “r” represents. Applicants respectfully submit that the recitation “r” was a typographical error which has been corrected in the claims as amended herein.

Accordingly, reconsideration and withdrawal of the rejection under 35 U.S.C. §112, second paragraph, is respectfully requested.

**III. THE ART REJECTIONS ARE OVERCOME**

Claims 1, 3, 4, 9, 11-14, 16-18, 26, 27, 29, 32, 33, 37, 46-50, 52, 53, 56, 57, 60, 63, 64, 67-70 and 75 were rejected under 35 U.S.C. §102(e) as allegedly being anticipated by U.S. Patent No. 6,548,067 to Seeman *et al.* Claims 1, 3, 4, 9, 11-14, 16-18, 26, 27, 29, 32, 33, 37, 46-

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49, 51-53, 56, 57, 60, 63, 64, 67-70 and 75 were rejected under 35 U.S.C. §103(a) as allegedly being unpatentable over U.S. Patent No. 6,548,067 to Seeman *et al.* in view of Neri *et al.* The rejections are respectfully traversed and will be addressed collectively.

It is respectfully asserted that a two-prong inquiry must be satisfied in order for a Section 102 rejection to stand. First, the prior art reference must contain all of the elements of the claimed invention. *See Lewmar Marine Inc. v. Barient Inc.*, 3 U.S.P.Q.2d 1766 (Fed. Cir. 1987). Second, the prior art must contain an enabling disclosure. *See Chester v. Miller*, 15 U.S.P.Q.2d 1333, 1336 (Fed. Cir. 1990). A reference contains an enabling disclosure if a person of ordinary skill in the art could have combined the description of the invention in the prior art reference with his own knowledge of the art to have placed himself in possession of the invention. *See In re Donohue*, 226, U.S.P.Q. 619, 621 (Fed. Cir. 1985).

Turning to the obviousness rejection, it is also respectfully asserted that it is well-settled that there must be some prior art teaching which would have provided the necessary incentive or motivation for modifying the reference teachings. *In re Laskowski*, 12 U.S.P.Q. 2d 1397, 1399 (Fed. Cir. 1989); *In re Obukowitz*, 27 U.S.P.Q. 2d 1063 (BOPAI 1993). Further, “obvious to try” is not the standard under 35 U.S.C. §103. *In re Fine*, 5 U.S.P.Q. 2d 1596, 1599 (Fed. Cir. 1988). And, as stated by the Court in *In re Fritch*, 23 U.S.P.Q. 2d 1780, 1783-1784 (Fed. Cir. 1992): “The mere fact that the prior art may be modified in the manner suggested by the Examiner does not make the modification obvious unless the prior art suggests the desirability of the modification.” Also, the Examiner is respectfully reminded that for the Section 103 rejection to be proper, **both the suggestion of the claimed invention and the expectation of success must be founded in the prior art, and not Applicants' disclosure.** *In re Dow*, 5 U.S.P.Q.2d 1529, 1531 (Fed. Cir. 1988).

Applicants respectfully assert that the requirements for a Section 102 or 103 rejection are not met by either of Seeman or Neri, either alone or in any combination.

The Office Action alleges that the present claims can be read as including complexes where the recognition peptide is capable of binding to the HLA class I molecule or fragment thereof, but has not done so, therefore rendering the recognition peptide separate from the complex itself. Office Action at 4-5. Applicants respectfully disagree.

Applicants respectfully assert that neither Seeman or Neri, either alone or in any combination, teach or suggest the present invention. Indeed, Seeman uses a single continuous

fusion protein in which each of the elements including the HLA are covalently joined. This is in contrast to the present invention which uses a two step biotin/streptavidin coupling system in order to join the elements of the complex together. This advantageously allows assembly of the complex *in vivo*, and allows a more modular nature of the system, for example allowing production of a common attaching means to be coupled to different HLA/recognition peptide elements, thereby simplifying use of the system for different applications. Seeman, however, requires a completely new fusion protein to be produced for each different application. This is overcome by the present invention.

Furthermore, Seeman relies on an alloreactive response for their technique. As is known by one of skill in the art, an alloreactive response is a relatively weak response generated by tissue mis-match/organ rejection/auto-immune type factors. In contrast, the present invention uses a recognition peptide in order to drive the response. Furthermore, the recognition peptide used is attached to an HLA, wherein the HLA is the patient's own type. Therefore, the present invention does not generate an alloreactive response since the HLA is type matched to the patient. More importantly, it is the recognition peptide which mediates the strong and aggressive response in the present invention. As no such recognition peptide is taught or suggested by Seeman, Seeman necessarily lacks a response that is as effective as that of the present invention.

In fact, there is no mention of peptides in Seeman. Seeman's HLA molecule is "empty", resulting in a system that does not work. In contrast, the system provided for by the present invention provides as an essential element of the invention a recognition peptide attached to the HLA molecule, which provides a strong and aggressive response, as described previously, and as taught in the specification in Examples 1 and 2 at pages 17-27. This is significant as Seeman provides no data.

Yet another difference between Seeman and the present invention is that Seeman's system cannot be used for T cell expansion. One of skill in the art would recognize that it is not feasible to expand alloreactive T cells *in vivo*. Secondly, Seeman's system cannot be used to expand therapeutically useful T cells either *in vivo* or *in vitro* due to the lack of recognition peptide and the alloreactive response which is garnered by Seeman. In contrast, the present invention is perfectly suited to therapeutic T cell expansion and success in such an endeavour has been demonstrated.

As evidence of the success of the present invention, enclosed are two articles which discuss results obtained using the present invention. First, Barber *et al* demonstrates the use of the present invention for *in vitro* immunological assays. Second, Savage *et al* demonstrates both the *in vitro* and *in vivo* activity of the antibody – HLA system of the present invention for targeting and killing tumour cells in mice.

As described above, Seeman fails to teach or suggest the present invention. Furthermore, nothing in Neri would overcome the deficiencies of Seeman, especially as there are no recognition peptides disclosed in Neri, and there are no HLA molecules disclosed in Neri. Hence, the combination of Seeman and Neri would still fall short of the present invention as the system would not be able to overcome the lesser, allereactive response of Seeman.

Accordingly, for all of the reasons set forth above, the present invention is novel and non-obvious over Seeman and Neri, either alone or in combination. Accordingly, reconsideration and withdrawal of the rejections under 35 U.S.C. §§ 102 and 103 are respectfully requested.

#### **REQUEST FOR INTERVIEW**

If any issue remains as an impediment to allowance, an interview, with supervisory review, e.g., with the Examiner, and the Examiner's SPE, is respectfully requested prior to issuance of any paper other than a Notice of Allowance. The Examiner is additionally respectfully requested to telephonically contact the undersigned to arrange a mutually convenient time and manner for the interview. The Examiner is also invited to telephonically contact the undersigned if there are any minor, formal issues that need resolving prior to issuance of a Notice of Allowance, with a view towards resolving such minor, formal issues via telephonic interview.

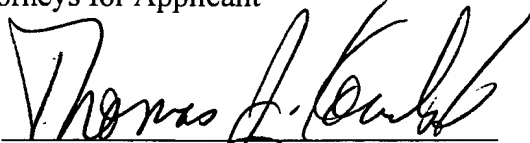
**CONCLUSION**

In view of these amendments and remarks, the application is in condition for allowance. Early and favorable reconsideration of the application, reconsideration and withdrawal of the objections and rejections, and prompt issuance of a Notice of Allowance are earnestly solicited.

Respectfully submitted,

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By:

A handwritten signature in black ink, appearing to read "Thomas J. Kowalski", written over a horizontal line.

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Research paper

# HLA class I mono-specific APCs and target cells: A method to standardise in vitro CD8<sup>+</sup> T cell expansion and functional assays

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## Abstract

The introduction of in vitro T cell expansion and assay methods that are robust and easy to use would be welcome in cancer vaccine and infectious disease research. By coating HLA class I –ve B cells with recombinant HLA class I peptide complexes, we are able to produce antigen presenting cells and target cells expressing a single defined antigen in the context of costimulatory and adhesion molecules. HLA class I mono-specific cells promoted the in vitro expansion of CMV epitope specific CD8<sup>+</sup> T cells from 0.03% to 30.6% in 2 weeks, which was comparable to using peptide-loaded dendritic cells. The HLA class I mono-specific cells were also shown to promote in vitro antigen specific T cell function in assays based on measuring cytokine production and cytolytic activity. HLA class I mono-specific cells are simple to prepare, can be used with any recombinant HLA class I allele/peptide combination and should provide a useful system for in vitro T cell expansion and functional analysis.

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**Keywords:** Antigen presenting cell; T cell; Assay; In vitro; Immunotherapy

## 1. Introduction

The effective monitoring of antigen specific T cell numbers and activity is central to the assessment of vaccination and other immunotherapy strategies in oncology and viral infections (Nagorsen et al., 2004). The introduction of recombinant HLA class I tetramers has greatly facilitated the rapid and accurate enumera-

tion of epitope specific CD8<sup>+</sup> T cells (Altman et al., 1996). However the assays that measure the functional abilities of T cells via cytokine production and release, granzyme B production or in vitro lytic activity are frequently difficult to perform, of limited reproducibility and subject to considerable background ‘noise’ (Keilholz et al., 2002). A further difficulty is that the epitope specific T cells within PBMC populations are often present at low frequencies, necessitating in vitro expansion prior to analysis.

In CD8<sup>+</sup> T cell expansion and functional assays, T cells interact with antigen presenting cells (APC) or target cells via the T cell receptor–HLA class I interface. The most

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widely used APCs or target cells are autologous B cells, autologous dendritic cells or partially HLA matched lymphoblastoid cell lines such as T2 cells (Scheibenbogen et al., 2000). However the use of these cells introduces a number of variables including, the ratio of T cells to APCs, the numbers of antigenic HLA class I/peptide complexes per cell and the presence of other HLA class I and II peptide complexes. These factors, combined with the lack of standardisation of any preceding in vitro expansion step, can lead to poor assay reproducibility. An approach that improves the specificity of assays, increases reproducibility and avoids the need for separate target cell lines for different HLA types would be a significant step forward.

We have previously described the use of a two step biotin–streptavidin anti-CD20 antibody targeting system to deliver recombinant HLA class I complexes to the surface of B cells. These complexes convert targeted B cells into highly effective APCs capable of producing significant expansion of peptide specific CD8<sup>+</sup> T cells (Stebbing et al., 2004). In addition, coating B cells with recombinant HLA class I complexes converts them to effective targets for cytolytic T cells of the appropriate antigen specificity (Savage et al., 2002). By using Daudi B cells which do not express HLA class I, this system can be adapted to produce a homogenous population of B cells coated with a defined amount of a single HLA class I/peptide complex for use as APCs or functional assay targets. In this report we have investigated the use of these cells, termed HLA class I mono-specific cells, for in vitro T cell expansion and functional assays.

## 2. Materials and methods

### 2.1. Preparation of peripheral blood mononuclear cells (PBMCs)

PBMCs were purified from the heparinized blood of healthy volunteer donors by Ficoll–Hypaque density gradient centrifugation. HLA class I genotyping was performed using PCR sequence-specific primers (Olerup SSP; Genovision, Alpha Helix). CMV status was determined by ELISA for CMV specific IgG antibodies.

### 2.2. Preparation of HLA class I monomers

In-house recombinant biotinylated HLA class I monomers were constructed as described previously (Whitelegg et al., 2005) using the HLA-A\*0201 binding immunogenic CMV peptide NLVPMVATV (pp65 protein 495–503), the HLA-B\*0702 binding immunogenic CMV peptide TPRVTGGGA (pp65 protein 417–425) or an irrelevant HLA-A\*0201 binding peptide TLWVDPYEV (BTG

protein 103–111). Additional proprietary biotinylated HLA-A\*0201 class I monomers incorporating the influenza peptide GILGFVFTL (MP protein 58–66), were purchased from ProImmune Ltd Oxford UK. The HLA class I monomers are referred to by the first three letters of the peptide.

### 2.3. Coating of Daudi cells with HLA class I monomers

HLA class I negative Daudi cells ( $1 \times 10^6$ /ml) were incubated with recombinant B9E9 single-chain Fv-streptavidin (ScFvSA) fusion protein (10 µg/ml) diluted in phosphate-buffered saline (PBS) for 1 h at 4 °C (Schultz et al., 2000). After washing in PBS, cells were incubated with biotinylated HLA class I monomers (0.5 µg/ml in PBS) for 30 min at room temperature. Coating with HLA class I monomer was verified by staining with FITC-conjugated anti HLA class I antibody W6/32 (Serotec) and analysis by flow cytometry.

### 2.4. In vitro expansion of CMV-specific CD8<sup>+</sup> T cells using Daudi cells coated with HLA class I monomers

Enrichment of CD8<sup>+</sup> cells from PBMCs was performed using anti CD8 antibody-coated magnetic beads (Miltenyi Biotec) according to manufacturer's instruction. Daudi cells ( $0.2 \times 10^6$ /ml) coated with HLA class I monomers were irradiated (100 Gy) and used to stimulate purified CD8<sup>+</sup> cells ( $1 \times 10^6$ /ml) in the presence of irradiated (30 Gy) autologous CD8 depleted PBMCs ( $1 \times 10^6$ /ml) added back as non-proliferating feeder cells in RPMI media with 10% FCS and IL-7 (R&D Systems) at 10 ng/ml. IL-2 (R&D Systems) at 20 U/ml was added from day 4. The cultured cells were re-stimulated every 7 days.

### 2.5. In vitro expansion of CMV-specific CD8<sup>+</sup> T cells using peptide loaded dendritic cells

To prepare monocyte-derived dendritic cells, PBMCs were cultured at  $4 \times 10^6$ /ml in RPMI media with 10% FCS in 6 well tissue culture dishes. Non-adherent cells were aspirated after 2–4 h. Adherent cells were cultured in 100 ng/ml IL-4 (R&D Systems) and 250 ng/ml GM-CSF (R&D Systems) and fresh media with cytokines was added every second day. After 6 days, dendritic cells were matured by overnight culture with 10 ng/ml TNF-α (R&D Systems) and 15 µg/ml poly (I:C) (Sigma-Aldrich). Maturation was confirmed by analysis of the CD80, CD83, CD86, CD11c and HLA-DR cell surface phenotype using fluorescent-labelled antibodies (BD Biosciences) and flow cytometry. Mature dendritic

cells were cultured with 20  $\mu\text{g}/\text{ml}$  synthetic peptide for 2 h and then washed to remove free peptide. Purified CD8<sup>+</sup> cells ( $1.5 \times 10^6/\text{ml}$ ) were stimulated with irradiated (30 Gy) peptide-loaded autologous dendritic cells ( $0.15 \times 10^6/\text{ml}$ ) in the presence of irradiated (30 Gy) autologous CD8 depleted PBMCs ( $1.5 \times 10^6/\text{ml}$ ) added back as non-proliferating feeder cells in Iscove's

modified Dulbecco's medium with 10% FCS containing 10 ng/ml IL-7 and 20  $\mu\text{g}/\text{ml}$  synthetic peptide. IL-2 at 20 U/ml was added from day 7 onwards. On day 12 and subsequently every 7 days adherent monocytes isolated from  $4 \times 10^6$  autologous irradiated (30 Gy) PBMCs were loaded with peptide and used to restimulate  $1.5 \times 10^6$  cultured T cells.

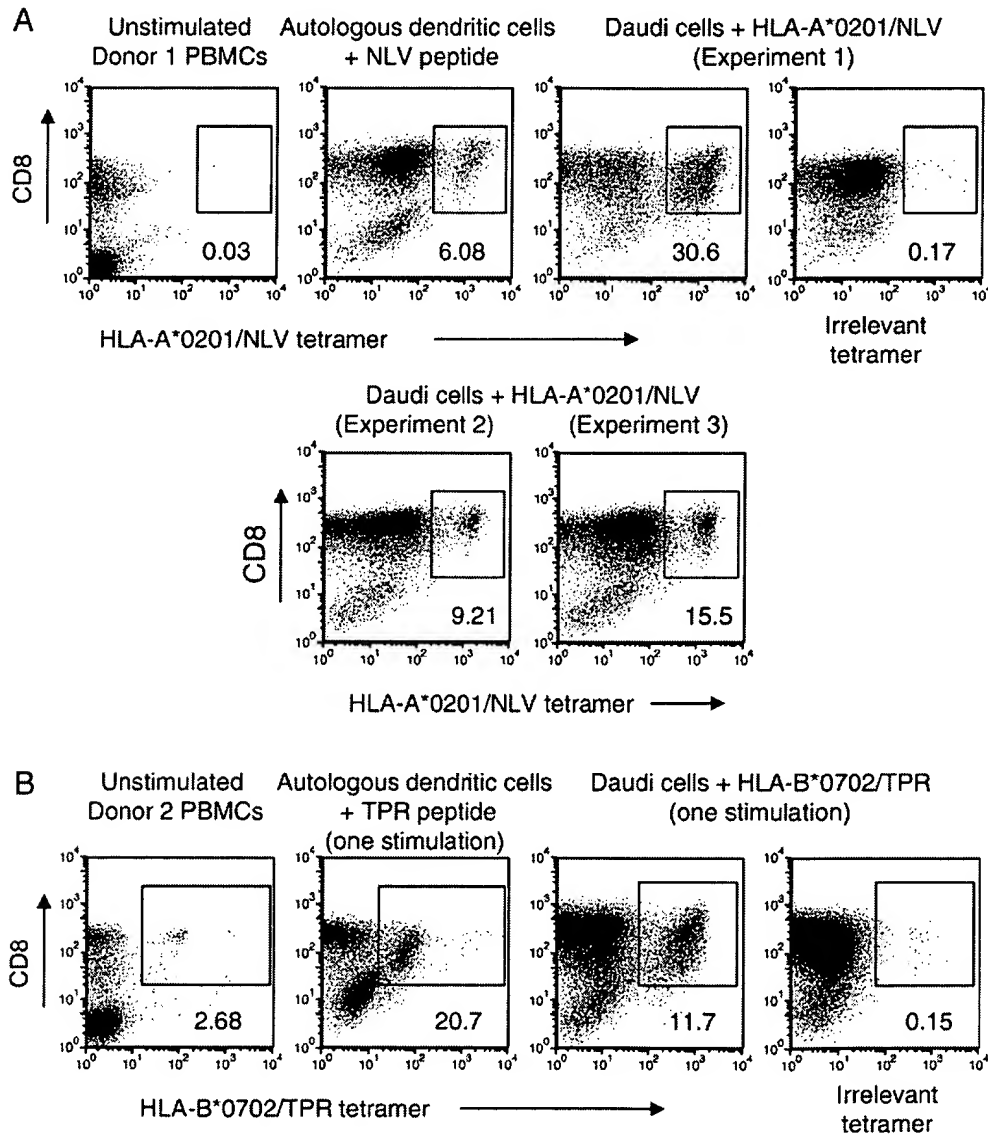


Fig. 1. In vitro stimulation using Daudi B cells coated with recombinant HLA class I monomers promotes expansion of CMV specific CD8<sup>+</sup> T cells comparable to peptide-loaded autologous dendritic cells. (A) Tetramer analysis of PBMCs from HLA-A\*0201 positive/CMV seropositive donor that have been stimulated in vitro with autologous peptide loaded dendritic cells or Daudi cells coated with HLA class I monomers to expand HLA-A\*0201/NLV specific CD8<sup>+</sup> T cells. Expansion using Daudi cells coated with HLA class I monomers was performed on three separate occasions. Cells expanded in the first experiment were also stained with an irrelevant tetramer. (B) Tetramer analysis of PBMCs from HLA-B\*0702 positive/CMV seropositive donor that have been expanded with autologous peptide loaded dendritic cells or Daudi cells coated with HLA class I monomers to expand HLA-B\*0702/TPR specific CD8<sup>+</sup> T cells. Cells were stained with FITC-conjugated anti CD8 antibody, PE-conjugated tetramer and PerCP-conjugated anti CD3 antibody. Live CD3<sup>+</sup> cells were gated and analyzed for expression of CD8 and tetramer binding. Results are expressed as the percentage of tetramer binding CD8<sup>+</sup> T cells.



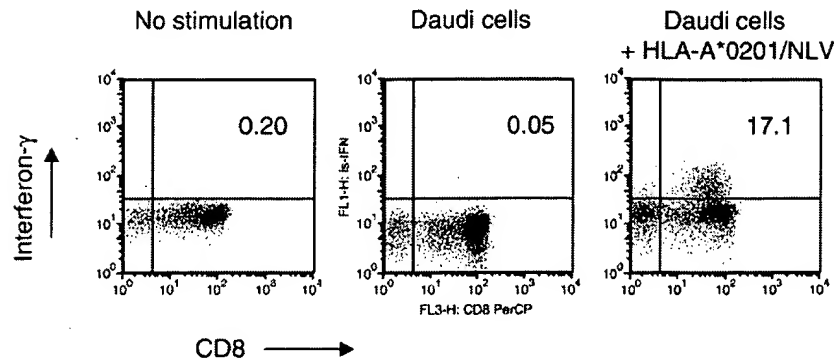


Fig. 2. Daudi cells coated with recombinant HLA class I monomers are effective APCs in a functional assay based on production of the cytokine interferon- $\gamma$ . CMV specific CD8 $^{+}$  T cells expanded in vitro using Daudi cells coated with recombinant HLA class I monomers were screened for production of cytokine after 4-h stimulation with Daudi B cells or Daudi B cells coated with HLA-A\*0201/NLV monomers. Cells were stained with PerCP-conjugated anti CD8 antibody and then intracellular staining was performed with FITC-conjugated anti interferon- $\gamma$  antibody. Results are expressed as the percentage of CD8 $^{+}$  cells producing cytokine and are representative of two separate experiments.

## 2.6. HLA class I tetramer staining, intracellular cytokine staining and flow cytometry

Biotinylated HLA class I monomers were converted into tetramers by adding PE-labelled streptavidin at a molar ratio of 4:1. Cells were incubated with tetramer (2  $\mu$ g HLA class I monomer per  $1 \times 10^6$  cells) at 37 °C for 30 min followed by saturating amounts of antibodies specific for CD8 conjugated to FITC and CD3 conjugated to PerCP at 4 °C for 30 min. To measure cytokine production,  $1 \times 10^6$  responder T cells were cultured with  $0.5 \times 10^6$  Daudi cells for 4 h with Brefeldin A (10  $\mu$ g/ml) added during the second hour of incubation. Cells were then stained with PerCP-conjugated antibody specific for CD8 at 4 °C for 30 min followed by intracellular staining performed with FITC-conjugated antibody specific for interferon-gamma according to manufacturer's instruction. All antibodies used were obtained from BD Biosciences. Cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences). Data was collected for a minimum of 100 000 live cells per sample and evaluated using FlowJo software (Tree Star). The phenotyping and functional assays with in vitro stimulated T cells were performed on days 5–7 after the last restimulation.

## 2.7. Chromium release assay

Standard Chromium-51 (51 Cr) release assays were performed using HLA class I monomer coated Daudi cells. Approximately  $10^6$  cells were labelled with 100  $\mu$ Ci of 51 Cr (Amersham Pharmacia, UK) for 1 h at 37 °C. Cells were coated with HLA class I monomers and plated at 3000 cells per well in U bottomed 96-well plates. Tissue culture media, effector cells (15,000 per well) or 5% Triton X-100

were added to a final volume of 200  $\mu$ l. After incubation for 4 h at 37 °C in a 5% CO $_2$  atmosphere, 50  $\mu$ l of supernatant were collected and 51 Cr content measured using a Microplate scintillation system. The specific lysis of target cells was calculated as: (experimental cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm)  $\times$  100. Spontaneous cpm was the measurement from target cells incubated in media alone and maximum cpm was the measurement from target cells incubated in Triton X-100.

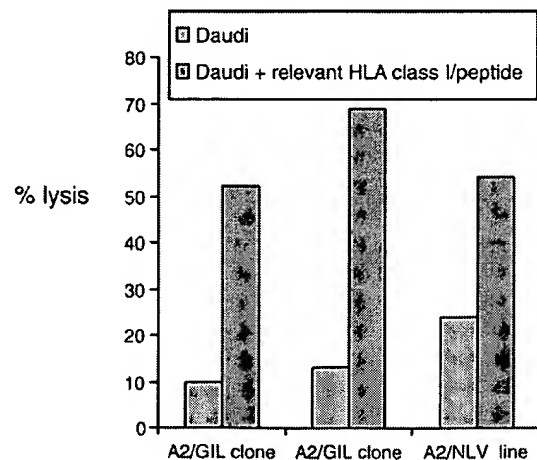


Fig. 3. Daudi cells coated with recombinant HLA class I monomers are effective targets in a functional assay based on cytolytic activity. A T cell clone specific for the Influenza antigen HLA-A\*0201/GIL and a T cell line specific for the CMV antigen HLA-A\*0201/NLV expanded from a donor by stimulation with Daudi cells coated with HLA-A\*0201/NLV monomers were screened for cytolytic activity. The target cells were either Daudi cells or Daudi cells coated with relevant HLA-A\*0201/peptide monomers in a 51 chromium release assay. Results are expressed as the percent specific lysis of the target cells and are representative of two separate experiments.

The CD8<sup>+</sup> T cell clone specific for influenza antigen HLA-A\*0201/GIL was obtained by limiting dilution from PBMCs infected *in vitro* with influenza.

### 3. Results

The ability of HLA class I mono-specific B cells to expand CMV antigen-specific T cells is demonstrated in Fig. 1A. Here CD8<sup>+</sup> T cells from donor 1 recognising the HLA-A\*0201/NLV epitope show expansion from 0.03% to 6.08% with two cycles of peptide loaded autologous dendritic cells, whilst two cycles of the HLA-A\*0201/NLV mono-specific Daudi cells produced expansion to 30.6%. The specificity of the CD8<sup>+</sup> T cell expansion produced by the HLA class I mono-specific cells is confirmed by the negative staining using a tetramer to HLA-A\*0201/TLW, which shows no significant binding. The reproducible expansion of HLA-A\*0201/NLV specific T cells from this donor by stimulation with HLA-A\*0201/NLV mono-specific Daudi cells was observed in two further experiments producing expansion to 9.2% and 15.5% after two cycles of *in vitro* stimulation. Fig. 1B shows the expansion of CD8<sup>+</sup> T cells recognising a different HLA class I/CMV peptide complex using HLA mono-specific cells carrying the HLA-B\*0702/TPR epitope. Here there is expansion from 2.68% to 20.7% using a single cycle of the dendritic cell method and to 11.7% using the HLA class I mono-specific cells.

The activity of HLA class I mono-specific cells as APCs in an intracellular cytokine assay (ICC) is demonstrated in Fig. 2. Here a T cell line with specificity for HLA-A\*0201/NLV expanded from the cells shown in Fig. 1A, was assayed for production of interferon- $\gamma$  by ICC staining using HLA class I mono-specific cells as the APCs. The unstimulated T cells produced background levels of cytokine at 0.2% and with native Daudi cells as APCs gave a similar background of 0.05%. However stimulation with HLA-A\*0201/NLV coated Daudi cells induced interferon- $\gamma$  production by 17.1% of the CD8<sup>+</sup> T cells. In addition to showing that HLA class I mono-specific cells can effectively induce an antigen specific functional response measured by the ICC assay, these results also confirm the functionality of T cells expanded *in vitro* using HLA class I mono-specific cells as APCs.

In Fig. 3 the activity of HLA class I mono-specific target cells in a series of chromium release assays is demonstrated. A cytotoxic T lymphocyte (CTL) clone recognising the influenza epitope HLA-A\*0201/GIL was used as the effector cell in two duplicate 4 h chromium release assays performed 4 weeks apart. The target cells were Daudi cells alone or Daudi cells coated with HLA-A\*0201/GIL complexes. The CTL produced 10% lysis of

the native Daudi B cells and 52% lysis of the cells coated with HLA-A\*0201/GIL on the first occasion, and 13% and 69% respectively on the second. Similar results are also demonstrated using the HLA-A\*0201/NLV specific CD8<sup>+</sup> T cell line expanded from donor 1 by stimulation with HLA class I monomer coated Daudi cells. These cells produced 54% lysis of the HLA-A\*0201/NLV coated cells compared to 24% of native Daudi cells 54%.

### 4. Discussion

In this study we have described a new system that offers improvements in the simplicity and utility of *in vitro* T cell expansion and functional assays. By attaching recombinant HLA class I complexes to HLA class I<sup>-ve</sup> B cells we are able to produce HLA class I mono-specific cells with identical specificity to their corresponding HLA class I tetramer, but with the full functional activity of an APC or target cell.

HLA class I mono-specific cells induced effective *in vitro* proliferation of antigen specific CD8<sup>+</sup> T cells. The expansion of CMV specific T cells specific for HLA-A\*0201/NLV and HLA-B\*0702/TPR demonstrated in Fig. 1 by stimulation with HLA class I mono-specific cells was comparable to that produced with conventional peptide pulsed dendritic cells. These two systems gave similar results, with both increasing the number of tetramer specific T cells 5–10 fold in 1–2 weeks. However, in practical terms, the dendritic cells needed for larger scale *in vitro* T cell expansion need to be obtained by leukapheresis from each individual donor and then be subjected to several days preparation prior to use. In contrast the HLA class I mono-specific cells take less than 1 h to prepare from Daudi cells growing in culture, use stable reagents and can be used for patients of any designated HLA class I allotype.

We have also demonstrated that HLA class I mono-specific cells can effectively stimulate responses in T cell functional assays. Antigen-specific responses clearly differentiated from background were observed in an ICC assay to measure cytokine production by T cells and in chromium release assays to measure T cell cytolytic activity. Functional assays have traditionally been performed using panels of peptide pulsed autologous APCs or partially HLA matched lymphoblastoid cell lines. Using the HLA mono-specific cells we demonstrated consistent levels of antigen-specific lysis by a T cell clone in two experiments performed 1 month apart. Whilst by their nature immunological analyses are subject to considerable variation, production of standardised targets eliminates one of the most difficult variables. Using more sensitive approaches such as flow cytometry

based lytic assays may help produce a higher degree of standardisation. Furthermore, the use of target cells that are both HLA class I and class II negative, such as K562 cells transfected with CD20 to permit coating with HLA class I monomers, would remove the possibility of any T cell/HLA class II interactions that may be responsible for the higher background lysis of Daudi target cells seen using the HLA-A\*0201/NLV T cell line (Fig. 3).

There have been previous attempts to produce standardized APCs based on use of HLA class I transfected cells, such as HLA –ve K562 (Britten et al., 2002). However this transfectant approach requires production and maintenance in culture of a panel of cell lines individually transfected with each of the required HLA alleles. In contrast, the HLA class I mono-specific cell approach requires only a single cell line that is produced to the required antigen specificity by coating with recombinant HLA class I/peptide complexes. The HLA class I mono-specific cells may also have advantages over new technologies that utilize artificial antigen presenting systems (Oelke et al., 2003; Schilbach et al., 2005). These systems allow the presentation of a single HLA class I/peptide complex alongside some co-stimulatory molecules and are effective in the expansion of T cells in vitro. However they are more complex to prepare and, because they are based on an inert core rather than a viable cell, they are not suitable for use in functional lytic assays.

In summary we have described a new approach using HLA class I mono-specific cells as APCs for promoting in vitro expansion of antigen specific CD8<sup>+</sup> T cells and for measuring CD8<sup>+</sup> T cell function. The cells express a single designated HLA/peptide combination, are available in unlimited numbers, are simple and quick to prepare and batches are easy to reproduce. These attributes should enable standardization of the APC platform used for the in vitro study of CD8<sup>+</sup> T cells.

## Acknowledgements

The work of LDB, SJ, AMEW and JAM was funded by The Anthony Nolan Trust.

## References

- Altman, J.D., Moss, P.A., Goulder, P.J., et al., 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274, 94.
- Britten, C.M., Meyer, R.G., Kreer, T., et al., 2002. The use of HLA-A\*0201-transfected K562 as standard antigen-presenting cells for CD8(+) T lymphocytes in IFN-gamma ELISPOT assays. *J. Immunol. Methods* 259, 95.
- Keilholz, U., Weber, J., Finke, J.H., et al., 2002. Immunologic monitoring of cancer vaccine therapy: results of a workshop sponsored by the Society for Biological Therapy. *J. Immunother.* 25, 97.
- Nagorsen, D., Scheibenbogen, C., Thiel, E., Keilholz, U., 2004. Immunological monitoring of cancer vaccine therapy. *Expert Opin. Biol. Ther.* 4, 1677.
- Oelke, M., Maus, M.V., Didiano, D., et al., 2003. Ex vivo induction and expansion of antigen-specific cytotoxic T cells by HLA-Ig-coated artificial antigen-presenting cells. *Nat. Med.* 9, 619.
- Savage, P., Cowburn, P., Clayton, A., et al., 2002. Anti-viral cytotoxic T cells inhibit the growth of cancer cells with antibody targeted HLA class I/peptide complexes in SCID mice. *Int. J. Cancer* 98, 561.
- Scheibenbogen, C., Romero, P., et al., 2000. Quantitation of antigen-reactive T cells in peripheral blood by IFN-gamma-ELISPOT assay and chromium-release assay: a four-centre comparative trial. *J. Immunol. Methods* 244, 81.
- Schilbach, K., Kerst, G., Walter, S., et al., 2005. Cytotoxic minor histocompatibility antigen HA-1-specific CD8<sup>+</sup> effector memory T cells: artificial APCs pave the way for clinical application by potent primary in vitro induction. *Blood* 106, 144.
- Schultz, J., Lin, Y., Sanderson, J., Zuo, Y., et al., 2000. A tetravalent single-chain antibody–streptavidin fusion protein for pretargeted lymphoma therapy. *Cancer Res.* 60, 6663.
- Stebbing, J., Gazzard, B., Patterson, S., et al., 2004. Antibody targeted MHC complex directed expansion of HIV-1 and KSHV-specific CD8 positive lymphocytes: a new approach to therapeutic vaccination. *Blood* 103, 1791.
- Whitelegg, A.M.E., Oosten, L.E.M., Jordan, S., et al., 2005. Investigation of peptide involvement in T cell allorecognition using recombinant HLA class I multimers. *J. Immunol.* 175, 1706.



## ANTI-VIRAL CYTOTOXIC T CELLS INHIBIT THE GROWTH OF CANCER CELLS WITH ANTIBODY TARGETED HLA CLASS I/PEPTIDE COMPLEXES IN SCID MICE

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A number of experimental antibody mediated cancer therapies aim to redirect cytotoxic T cells (CTLs) of non-tumour specificity to cancer cells. It has been previously demonstrated that cancer cells targeted with recombinant HLA-class I/viral peptide complexes via antibody delivery systems can be killed by virus specific CTLs. This novel therapeutic system has been developed with a simple pre-clinical model using the recombinant anti-CD20 B9E9 scFvSA fusion protein to target HLA-A2/peptide complexes to CD20 +ve Daudi lymphoma cells. *In vitro* data confirmed that, although binding of the B9E9 scFvSA fusion protein alone to Daudi cells had no effect on their growth, effective CTL mediated killing of Daudi cells could be achieved by targeting with B9E9 scFvSA and recombinant HLA-A2/MI complexes at dilutions as low as 100 pg/ml. In contrast the free HLA-A2/MI complexes only significantly inhibited CTL activity at concentrations in excess of 100 ng/ml. The *in vivo* tumour protection assays in SCID mice demonstrated that only 1 of the 4 mice that received anti-HLA-A2/MI CTLs and Daudi cells targeted with the B9E9 scFvSA fusion protein and HLA-A2/MI complexes developed a tumour. In contrast in the control mice that received CTL and native Daudi cells all 4 developed tumours, as did all 4 that received targeted Daudi cells but no CTLs. Similar results were obtained in a parallel experiment using Daudi cells targeted with B9E9 scFvSA and HLA-A2/BMLF1 complexes and a CTL line to HLA-A2/BMLF1. The demonstration of *in vivo* activity for targeted HLA class I/peptide complexes combined with anti-viral T cells, supports the further clinical development of the system where it may be combined with autologous CTLs produced by vaccination or *ex vivo* expansion.

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The production of a specific and effective anti-tumour CTL response is one of the central aims of cancer immunotherapy research.<sup>1</sup> Although this approach may develop as an effective therapy for some malignancies, difficulties with the specificity and level of expression of tumour peptides in tumour cells,<sup>2</sup> the frequency of down regulation of HLA class I expression in cancer cells<sup>3</sup> and the difficulty in upregulating the CTL response to these epitopes may limit clinical effectiveness.

A number of alternative immunotherapy strategies have been developed that aim to use the effector functions of the cellular immune system but using CTLs of non-tumour specificity redirecting them to cancer cells via antibody based systems. These methods include bispecific antibodies,<sup>4</sup> antibody-superantigen fusion proteins<sup>5</sup> and antibody cytokine fusion proteins.<sup>6</sup> It has been demonstrated previously that T cells of anti-viral specificity can kill cancer cells via antibody targeted delivery of recombinant MHC class I/peptide complexes used either as monomers<sup>7</sup> or as tetramers.<sup>8</sup>

The B cell surface antigen CD20 serves as a model target for the delivery of HLA class I/peptide complexes. It is expressed on most

B cell malignancies, has minimal internalization with bound antibody remaining immobilized on the cell surface for a number of days.<sup>9–11</sup> The therapeutic use of monoclonal antibodies to CD20 has now become established as routine in B cell lymphoma.<sup>12</sup> As a further development from the use of intact monoclonal antibodies either in native form or radiolabeled, a number of recombinant antibody fragments have been developed. The recently described anti-CD20 tetraivalent B9E9 scFvSA fusion protein is currently being investigated as a targeting system used with radiolabeled biotin in the radioimmunotherapy of lymphoma.<sup>13</sup> To study the ability of human CTLs of anti-viral specificity to interact with tumour cells targeted with HLA-A2/peptide complexes in a physiological setting, a pre-clinical model has been developed. Severe combined immunodeficient (SCID) mice are able to support functional human CTLs for varying periods depending on the degree of cytokine support<sup>14,15</sup> whilst the human B cell lymphoma Daudi line grows as a xenograft in these mice without further routine immunosuppression and has been used in a variety of therapeutic systems.<sup>16,17</sup>

For the next stage in the development of this therapeutic strategy we have examined the *in vivo* interaction of human anti-viral CTLs and HLA class I targeted Daudi lymphoma cells in simple tumour protection experiments.

### MATERIAL AND METHODS

#### Cell lines, mice and antibodies

Clone 25 is a human CD8<sup>+</sup>, TCR VB17<sup>+</sup> CTL clone specific for HLA-A\*0201 and influenza M1<sub>58–66</sub>. This clone was obtained by limiting dilution from PBMCs infected *in vitro* with influenza A. Clone 25 was propagated with fortnightly restimulations with irradiated allogeneic PBMC, PHA (0.5 µg/ml) and IL-2 (200 u/ml). Aliquots of 2 × 10<sup>6</sup> Clone 25 CTLs were cryopreserved and thawed for experimental use.

A human anti HLA-A2-BMLF1 CTL line documented to be 40% specific for HLA-A2/BMLF1 by tetramer analysis was cultured in RPMI with 10% AB serum and antibiotics.

The Daudi CD 20 +ve human B cell lymphoma cell line that is deficient for the expression of HLA class I<sup>18</sup> and the CIR-A2<sup>19</sup> HLA-A and -B negative human B-lymphoblastoid cell line transfected with the gene for HLA-A2 were cultured in RPMI media

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supplemented with 10% FCS and antibiotics in a 37°C incubator with 5% CO<sub>2</sub>.

SCID mice aged 6–8 weeks were maintained in sterile conditions at the ICRF animal facility.

The B9E9 scFvSA fusion protein contains the single-chain variable region of the murine IgG2a anti-CD20 murine antibody B9E9 fused to the genomic streptavidin of *Streptomyces avidinii*. B9E9 fusion is secreted into the periplasm of genetically engineered *E. coli* as monomeric subunits (43,400 Da) that spontaneously fold into soluble tetramers with a molecular weight of 173,600 Da. The 4 antigen-binding and biotin-binding sites of the fusion protein retain the functional capabilities of the parent molecules.<sup>13</sup>

#### Action of B9E9 scFvSA on Daudi cell growth

Daudi cells were washed in PBS and incubated with dilutions of B9E9 scFvSA in PBS for 1 hr at room temperature. After 2 washes cells were re-suspended in 5 ml of tissue culture media and incubated at 37°C in a 5% CO<sub>2</sub> atmosphere. The proliferation of the antibody treated cells and control was assessed by sequential counts of viable cells using Trypan blue exclusion and a hemocytometer.

#### Effect of HLA dilutions on in vitro CTL mediated lysis

Standard Chromium release assays were performed using targeted Daudi cells. Approximately 10<sup>6</sup> cells were labeled with 100 uCi of <sup>51</sup>Cr (Amersham Pharmacia, UK) for 1 hr at 37°C. After washing in PBS cells were incubated with B9E9 scFvSA at 10 µg/ml for 1 hr at room temperature. After washing cells were incubated with dilutions of HLA-A2/M1 complexes diluted in PBS for 1 hr at 4°C. After 2 further washes the cells were plated out at 3,000 cells per well in U bottomed 96-well plates. Tissue culture media, CTLs at an E:T ratio of 5:1 or 5% Triton X-100 were added to a final volume of 200 µL. After incubation for 4 hr at 37°C in a 5% CO<sub>2</sub> atmosphere 50 µL of supernatant was collected and radioactive counts determined using a Microplate scintillation system. The specific lysis was calculated as:

$$\% \text{ lysis} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{maximum cpm} - \text{spontaneous cpm}} \times 100$$

The spontaneous release was measured from the cells incubated in media alone, the maximum release was measured from the cells incubated in Triton X.

#### Flow cytometry analysis

Flow cytometry was performed on Daudi cells targeted with B9E9 scFvSA fusion protein and HLA-A2/M1 prepared as above. Samples of cells were washed in PBS and then incubated with FITC labeled anti-MHC class I (1 µg/ml, 30 min at RT) (Ansell, Nottingham, UK) and analyzed by flow cytometry on a Becton Dickinson FACscan with a 15 mW 488 nm air-cooled argon laser and standard band pass filters for FI-1 (530/30 nm), FI-2 (585/42 nm) and FI-3 (625 nm). Data acquisition and analysis was performed using CellQuest software (BD).

#### Effects of free HLA class I/peptide complexes on CTL viability and function

Dilutions of recombinant the monomeric HLA-A2/BMLF1 complex were made and added to 4 × 10<sup>5</sup>/ml of the anti-A2/BMLF1 CTL line growing in 2 ml vol in 24-well tissue culture plates. Cells were incubated overnight for 16 hr and then viable cell numbers were estimated by Trypan blue exclusion counting using a hemocytometer.

The functional activity of CTLs treated overnight with free HLA-A2/BMLF1 complexes was examined in a 4-hr Chromium release assay. This experiment used the protocol described above but employed CIR-A2 cells pulsed with the BMLF1 (10 mM) peptide as targets and the CTLs at E:T ratios of either 1:1 or 5:1.

#### In vivo tumour protection assays

**Experiment 1: CTL clone 25 to HLA-A2/M1.** Healthy male SCID mice 6–8 weeks of age were used in the first tumour protection assay. Four mice were used in each of the 3 groups (A–C). Mice in Group A were injected IP with 3 × 10<sup>6</sup> clone 25 cells in 0.2 ml of sterile PBS on Day 1. On Day 2, 1 × 10<sup>6</sup> Daudi cells targeted sequentially *ex vivo*, with B9E9 scFvSA (10 µg/ml) and HLA-A2/M1 (0.5 µg/ml) were injected IP in 0.2 ml of sterile PBS. Mice in Group B were injected IP with 3 × 10<sup>6</sup> clone 25 cells in 0.2 ml of sterile PBS on Day 1. On Day 2, 1 × 10<sup>6</sup> native Daudi cells were injected IP in 0.2 ml of sterile PBS. Mice in Group C were injected with 0.2 ml of sterile PBS on Day 1. On Day 2, 1 × 10<sup>6</sup> Daudi cells targeted sequentially, *ex vivo*, with B9E9 scFvSA (10 µg/ml) and HLA-A2/M1 (0.5 µg/ml) were injected IP in 0.2 ml of sterile PBS. The mice in all 3 groups of Experiment 1 received IP injections with human IL-2 (Chiron, UK) 2,500 U in 0.1 ml PBS daily on Days 1–3. Following these procedures the mice were maintained in sterile conditions and monitored for tumour development. Experiment 1 mice were sacrificed at Day 60 and assayed for tumour development. The identity of tumour deposits as B cell lymphoma was confirmed by immunocytochemistry.

**Experiment 2: CTL line to HLA-A2/BMLF1.** Healthy female SCID mice 6–10 weeks of age were used in the second tumour protection assay. Four mice were used in each of the 4 groups (1–4). On Day 1 mice in Group 1 were injected IP with 1 × 10<sup>7</sup> of the anti-BMLF1 CTL line. At a separate IP site 1 × 10<sup>6</sup> Daudi cells targeted sequentially, *ex vivo*, with B9E9 scFvSA (10 µg/ml) and HLA-A2/BMLF1 (0.5 µg/ml) were injected IP in 0.2 ml of sterile PBS. On Day 1 mice in Group 2 were injected IP with 1 × 10<sup>6</sup> Daudi cells targeted sequentially, *ex vivo*, with B9E9 scFvSA (10 µg/ml) and HLA-A2/BMLF1 (0.5 µg/ml) were injected IP in 0.2 ml of sterile PBS. On Day 1 mice in Group 3 were injected IP with 1 × 10<sup>7</sup> of the anti-BMLF1 cell line. At a separate IP site 1 × 10<sup>6</sup> native Daudi cells were injected IP in 0.2 ml of sterile PBS. On Day 1 mice in Group 4 were injected IP with 1 × 10<sup>6</sup> native Daudi cells in 0.2 ml of sterile PBS. Following these procedures the mice were maintained in sterile conditions and monitored for tumour development. Experiment 2 mice were sacrificed at Day 43 and assayed for tumour development.

## RESULTS

#### Effects of B9E9 scFvSA binding on Daudi cell kinetics in vitro

To investigate the potential effects on cell growth kinetics from the binding of the B9E9 scFvSA fusion protein to the Daudi lymphoma cells a simple *in vitro* proliferation study was performed. The results from this are shown in Figure 1. The results demonstrate that within the 3 day experimental period there is no significant effect on growth by an incubation with B9E9 scFvSA at either 1 µg/ml or 10 µg/ml.

#### Dose response of HLA binding concentration measured by flow cytometry and Chromium release assay

Figure 2 shows the effects on flow cytometry signal resulting from changes in the concentration of the HLA class I/peptide complex incubated with the B9E9scFvSA targeted Daudi cells. The results indicate that after a 1 hr incubation the presence of recombinant HLA class I complexes could be detected on the surface of the HLA class I –ve Daudi cells after incubation concentrations of 10–100 ng/ml or greater.

The results of the functional assay using a 4-hr Chromium release assay with clone 25 at an E:T ratio of 5:1 are shown in Figure 3. The positive results on this dose response graph extend over wider range than the flow cytometry result with significant lysis occurring after incubations with concentrations of free HLA-A2/M1 of 100 pg/ml or greater.

**Effects of free recombinant HLA class I peptide complexes on CTL function.** Figure 4a demonstrates the effects of overnight incubation with free HLA-A2/BMLF1 on the viability and prolif-

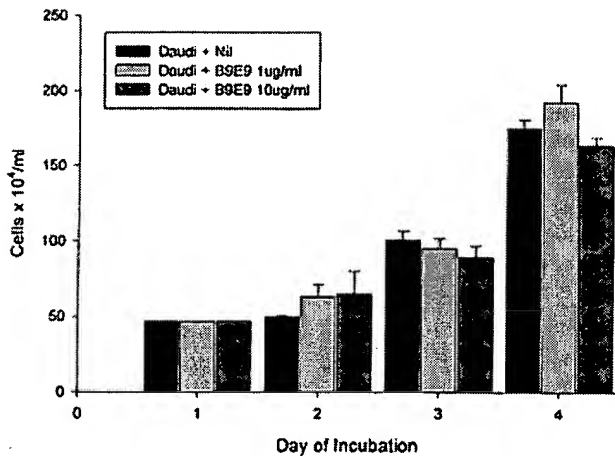


FIGURE 1 – The effects of B9E9 sfvScSA binding to the growth of Daudi cells *in vitro*. The results shown are for 3 days culture after a 1-hr exposure to dilutions of B9E9 sfvScSA.

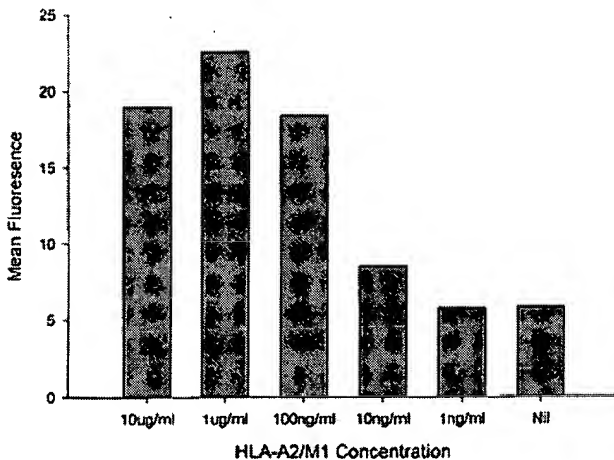


FIGURE 2 – Binding of HLA-A2/M1 complexes to Daudi cells targeted with B9E9 sfvScSA measured by flow cytometry using FITC conjugated anti-MHC class I.

eration of the CTL line to this specificity. Incubation with complexes at 10  $\mu$ g/ml caused a 50% reduction in total viable cell numbers. At 1  $\mu$ g/ml there was a reduction in viable cell numbers of 25%. At the lower concentrations of HLA-A2/BMLF1 complexes of 100 ng/ml or below the effect on viable cell number appeared to rapidly diminish. The functional activity of cells pre-treated with the HLA-A2/BMLF1 complexes is shown in Figure 4b. At 10  $\mu$ g/ml and 1  $\mu$ g/ml there was only minimal lytic activity against the BMLF1 peptide pulsed CIR-A2 cells. At the lower concentrations of 100 ng/ml or below the lytic activity was comparable to that of the untreated control cells. The results of the loss of lytic activity and the reduction in cell numbers at the concentrations of 1  $\mu$ g/ml and 10  $\mu$ g/ml are consistent with loss of virtually all the specific anti-HLA-A2/BMLF1 CTLs within this line that was documented to contain approximately 40–50% HLA-A2/BMLF1 tetramer positive cells.

#### *In vivo* tumour protection assay

The ability of anti-viral CTLs to interact with cancer cells targeted with the HLA-class I peptide complexes was examined in tumour protection assays in SCID mice. The results of the tumour

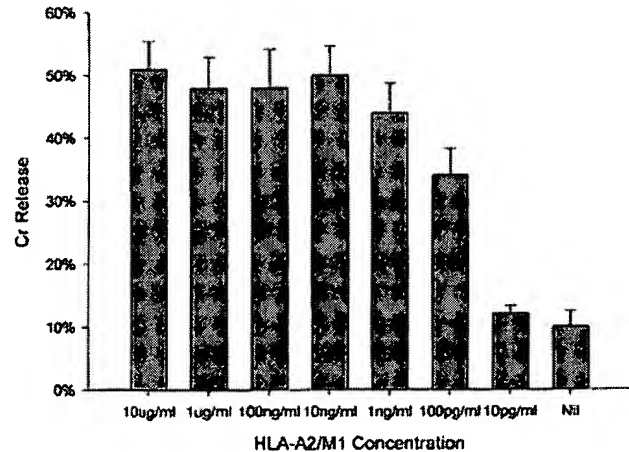


FIGURE 3 – Lysis of Daudi cells targeted with B9E9 sfvScSA and HLA-A2/M1 complexes in a 4-hr chromium release assay using the anti-HLA-A2/M1 specific clone 25 at E:T ratio of 5:1.

protection assay using clone 25 to HLA-A2/M1 displayed in Table I demonstrate that although there were measurable tumours in all the mice of the 2 control groups, tumour was only found in 1 of the group that received both the anti-HLA-A2/M1 CTLs and the targeted tumour cells. The results of a similar SCID mouse tumour protection assay using targeted HLA-A2/MBMLF1 complexes and a polyclonal CTL line to HLA-A2/BMLF1 are shown in Table II. These results also show a clear differentiation between experimental and control groups, with only 1 of the 4 experimental mice developing a tumour whereas all 12 in the 3 groups of control mice developed significant tumours by Day 43.

#### DISCUSSION

The potential use of the cellular immune system to selectively attack cancer cells is being developed in a number of different approaches. An increasing number of tumour associated peptides that may serve to immunologically distinguish cancer from normal cells have been identified.<sup>20,21</sup> It is possible, however, that low levels of peptide expression and the down regulation of HLA class I expression found in some tumour cells may limit the effectiveness of attempts to upregulate CTL responses against tumour cells.

Although defining effective tumour specific CTL targets may hold difficulties, many tumour cells bear tumour specific or tumour associated cell surface antigens that can be bound by monoclonal antibodies<sup>22</sup> a number of which are now used in clinical practice.<sup>23</sup> In contrast to the difficulties in producing CTLs specific for tumour cells the production of large numbers of CTLs reactive with viral epitopes is more straightforward, either by infection or vaccination *in vivo* or *ex vivo* by specific antigenic stimulation.

We have described previously the concept of using antibody targeted HLA class I/peptide complexes as a method to redirect the lytic action of CTLs of anti-viral specificity to tumour cells.<sup>7</sup> The preliminary *in vitro* work in that study, supported by parallel work using antibody targeted HLA class I tetramers,<sup>8</sup> demonstrated that a range of antibody targeted tumour cells could be effectively killed by anti-viral CTLs.

Our current study expands on that work with preliminary *in vivo* data using a system reduced to 2 targeting steps by the use of the B9E9 scFvSA fusion protein. This tetraivalent recombinant molecule has recently been shown to be highly effective in the radio-immunotherapy treatment of CD20+ve B cell lymphoma in an animal model<sup>13</sup> and is currently in phase I trials for the treatment of relapsed lymphoma.

The binding of monoclonal antibodies to CD20 appears to have differing effects depending on the identity of the antibody and cell

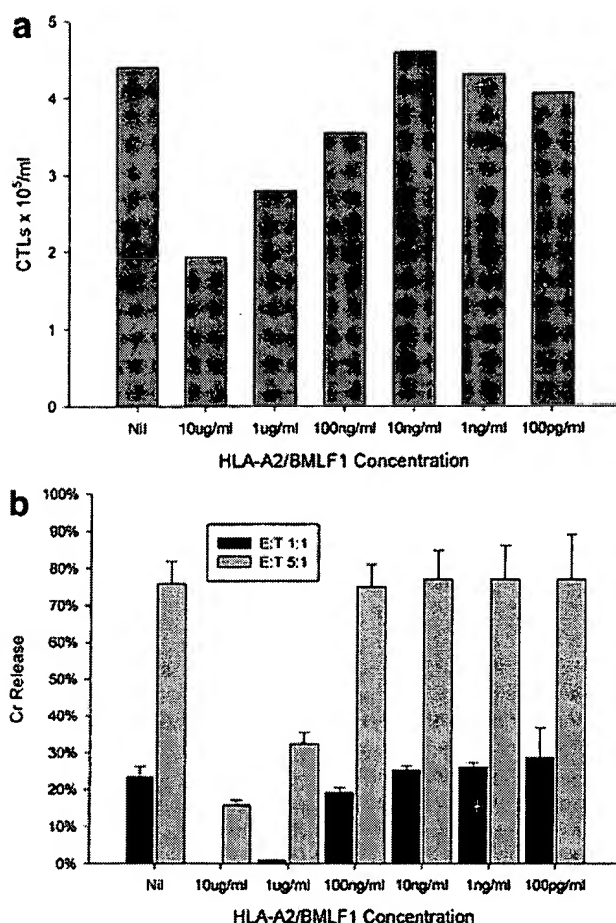


FIGURE 4—(a) The viability of CTLs reactive with HLA-A2/BMLF1 after 16 hr incubation with varying concentrations of free HLA-A2/BMLF1. CTL viability was determined by Trypan blue exclusion. (b) The lytic activity of CTLs reactive with HLA-A2/BMLF1 after 16 hr pre-incubation with concentrations of free HLA-A2/BMLF1. CTLs were then used in a 4-hr chromium release assay at E:T ratios of 5:1 and 1:1 with CIR-A2 target cells pulsed with BMLF1 peptide.

TABLE 1—RESULTS OF THE *IN VIVO* TUMOUR PROTECTION ASSAY IN SCID MICE USING HLA-A2/M1 TARGETED DAUDI CELLS AND THE CTL CLONE 25 TO HLA-A2/M1. MICE WERE SACRIFICED AND TUMOURS WEIGHED ON DAY 60

Group A Clone 25 Targeted Daudi	Group B Clone 25 Native Daudi	Group C PBS Targeted Daudi
Tumour 2.4g	Tumour 5.64g	Tumour 3.78g
No Tumour	Tumour 0.66g	Tumour 1.50g
No Tumour	Tumour 1.54g	Tumour 3.06g
No Tumour	Tumour 2.36g	Tumour 3.47g

line examined.<sup>24,25</sup> Before conducting the *in vivo* experiments we examined the effects on growth of binding of B9E9 scFvSA to Daudi cells. The results shown in Table I demonstrate that B9E9 scFvSA at concentrations of 1 µg/ml and 10 µg/ml gave no significant effect on cell growth. These results supporting the lack of induction of apoptosis produced by the parent immunoglobulin binding to Daudi cells in culture.<sup>25</sup>

In our previous study, saturating concentrations of all of the 3 steps in the targeting system were employed. To investigate the

TABLE 2—RESULTS OF THE *IN VIVO* TUMOUR PROTECTION ASSAY IN SCID MICE USING HLA-A2/BMLF1 TARGETED DAUDI CELLS AND THE CTL LINE TO HLA-A2/BMLF1. MICE WERE SACRIFICED AND TUMOURS WEIGHED ON DAY 43

Group 1 BMLF1 CTLs Targeted Daudi	Group 2 No CTLs Targeted Daudi	Group 3 BMLF1 CTLs Native Daudi	Group 4 No CTLs Native Daudi
Tumour 1.05g	Tumour 2.58g	Tumour 3.95g	Tumour 2.94g
No Tumour	Tumour 1.75g	Tumour 6.3g	Tumour 4.99g
No Tumour	Tumour 2.01g	Tumour 3.68g	Tumour 3.64g
No Tumour	Tumour 3.01g	Tumour 2.36g	Tumour 2.61g

effects of varying the binding concentrations of HLA class I/peptide complex, a dose response experiment was performed *in vitro* analyzing the level and activity of HLA class I bound by flow cytometry and by the functional Chromium release assay. The results in Figures 2 and 3 demonstrate that although the positive flow cytometry results are lost below HLA concentrations of 10 ng/ml, significant T cell mediated lysis of tumour cells can occur with significantly lower concentrations of the biotinylated HLA class I/M1 complex. Only at concentrations below 100 pg/ml does the degree of CTL mediated lysis reduce, with some activity maintained down to 10 pg/ml. These concentrations can be contrasted with the serum levels of recombinant protein achieved in therapy with the anti-CD20 monoclonal antibody Rituximab, which are in the order of 50–500 µg/ml.<sup>26</sup> The low concentrations of HLA class I/peptide required for activity in this system being a reflection of the high affinity reaction between streptavidin and biotin and the relatively low numbers of bound HLA class I/peptide complexes needed to effect CTL action. This ability of anti-viral CTLs to recognize and lyse target cells bearing relatively few targeted HLA-class I/peptide complexes may be of importance clinically. *in vivo*, the difficulties of tumour access, combined with the continual loss of targeted HLA class I peptide complexes by antigenic shedding and degradation, would make it unlikely that saturated binding could be achieved even with a molecule that is expected to be non-toxic in the clinical setting.

A further consideration for *in vivo* applications is the potential effects of the soluble monomeric HLA class I/peptide complex on T cell function before localization. The data in Figure 4a,b demonstrates that concentrations of free HLA-A2/BMLF1 complexes of 1 µg/ml and 10 µg/ml have a major impact on the CTL viability and function with a near total loss of activity. In contrast overnight exposure to HLA-A2/BMLF1 complex concentrations of 100 ng/ml and below appears to produce no significant effect on either CTL viability or function.

The potential inhibitory and pro-apoptotic effects of free HLA-class I has previously been documented in a number of studies that have focused on alloreactive CTLs. Solubilized full length transmembrane HLA class I, that can form multimers, caused apoptosis and inhibition of alloreactive T cell function at concentrations above 1 µg/ml,<sup>27</sup> whereas recombinant truncated HLA class I molecules, that are unable to multimerize, have also been demonstrated to affect CTL activity in an alloreactive model, at concentrations of 2 µg/ml and above.<sup>28</sup> The data in our current study suggests that similar effects may occur with the free biotinylated HLA-A2/peptide complexes that we aim to use therapeutically. It is possible, however, that the potential negative effects from HLA-A2/peptide complexes on endogenous T cell function may be avoidable, as the concentration needed to effectively target cells at 100 pg/ml is 3 orders of magnitude lower than those detrimental to CTL function.

The ability of this system to function in a physiological setting was examined in a SCID mouse pre-clinical model. Although these experiments used target cells that were treated *ex vivo* before



administration, they confirm that if HLA class I/complexes can be delivered to target cells they can be effectively lysed by anti-viral T cells *in vivo*.

The first experiment examined the ability of a T cell clone against HLA-A2/M1 to protect against inoculation with Daudi cells targeted with HLA-A2/M1 complexes via the B9E9 sfvScSA fusion protein. Of the animals pre-treated with the anti-HLA-A2/M1 CTL clone 25 on Day 1 and then injected with targeted Daudi cells on Day 2, only 1 of the 4 developed a tumour. In contrast the control groups, of mice either pre-treated with clone 25 but receiving native Daudi cells or mice with no CTL pre-treatment receiving targeted Daudi cells all 4 of each group developed tumours.

Similar results were obtained using a CTL line prepared from donor PBLs that contained approximately 40% of CTLs reactive to HLA-A2/BMLF1. These cells, when coinjected with the targeted Daudi cells, produced protection in 3 of the 4 mice whereas large tumours grew in all the mice in each of the 3 control groups.

Although experiments in SCID mice particularly those using CTL clones may be distant from the clinical situation, the ability of anti-viral CTLs to protect against tumour challenge with HLA class I targeted cells has been clearly demonstrated in these experiments.

The use of streptavidin for *in vivo* targeting systems raises concerns regarding the potential development of antibody responses to this immunogenic protein. Although these responses

have been documented in clinical trials<sup>30,31</sup> that use similar delivery systems, the clinical impact of these responses is at present unclear. A number of methods are being explored that could reduce the clinical impact of the immunogenicity of streptavidin, however, these include PEGylation,<sup>32</sup> protein engineering to remove immunogenic epitopes<sup>13</sup> or the use of alternative binding partners such as calmodulin and a calmodulin binding peptide.<sup>33</sup>

The low toxicity of the targeting antibody and probable low toxicity of the recombinant HLA class I peptide complexes suggest that it may be feasible to deliver sufficient molecules to target cells to permit effective CTL activity. It is also possible that the HLA class I stability may be improved by the production of single chain recombinant versions.<sup>29</sup>

The question of how to deliver sufficient CTLs to effect the lysis of the targeted cells will need further consideration, however it is encouraging that the anti-A2/BMLF1 line that is a mixed population containing 40% specific cells was able to produce effective tumour protection in the SCID model. It may be possible that pre-existing CTLs<sup>34</sup> or those upregulated by obtained by infection,<sup>35</sup> or vaccination may be sufficient or that approaches modelled on the administration of CTLs expanded *ex vivo*, such as described for the treatment of EBV associated lymphoma<sup>36,37</sup> may be more appropriate. We are currently assessing the potential role of these various approaches before the development of Phase I trials in melanoma and lymphoma.

## REFERENCES

- Rosenberg SA. Development of cancer immunotherapies based on identification of the genes encoding cancer regression antigens. *J Natl Cancer Inst* 1996;88:1635-44.
- Neumann E, Engelsberg A, Decker J, et al. Heterogeneous expression of the tumour-associated antigens RAGE-1, PRAME and glycoprotein 75 in human renal cell carcinoma: candidates for T cell based immunotherapies? *Cancer Res* 1998;58:4090-5.
- Cormier JN, Panelli MC, Hackett JA, et al. Natural variation of the expression of HLA and endogenous antigen modulates CTL recognition in an *in vitro* melanoma model. *Int J Cancer* 1999;80:781-90.
- Van Ojik HH, Valerius T. Preclinical and clinical data with bispecific antibodies recruiting myeloid effector cells for tumour therapy. *Crit Rev Oncol Hematol* 2001;38:47-61.
- Nielsen SE, Zeuthen J, Lund B, et al. Phase I study of single escalating doses of a superantigen-antibody fusion protein (PNU-214565) in patients with advanced colorectal or pancreatic carcinoma. *J Immunother* 2000;23:146-53.
- Penichet ML, Morrison SL. Antibody-cytokine fusion proteins for the therapy of cancer. *J Immunol Methods* 2001;248:91-101.
- Ogg GS, Dunbar PR, Cerundolo V, et al. Sensitization of tumour cells to lysis by virus-specific CTL using antibody-targeted MHC class I peptide complexes. *Br J Cancer* 2000;82:1058-62.
- Robert B, Guillaume P, Luescher I, et al. Antibody-conjugated MHC class I tetramers can target tumour cells for specific lysis by T lymphocytes. *Eur J Immunol* 2000;30:3165-70.
- Press OW, Appelbaum F, Ledbetter JA. Monoclonal antibody 1F5 (anti-CD20) serotherapy of human lymphomas. *Blood* 1987;69:584-91.
- Press OW, Farr AG, Borroz KI, et al. Endocytosis and degradation of monoclonal antibodies targeting human B cell malignancies. *Cancer Res* 1989;49:4906-12.
- Hanna R, Ong GL, Mattes MJ. Processing of antibodies bound to B cell lymphomas and other hematological malignancies. *Cancer Res* 1996;56:3062-8.
- Hainsworth JD. Rituximab as first line systemic therapy for patients with low-grade lymphoma. *Semin Oncol* 2000;27:25-9.
- Schultz J, Lin Y, Sanderson J, et al. A tetravalent single-chain antibody-streptavidin fusion protein for pretargeted lymphoma therapy. *Cancer Res* 2000;60:6663-9.
- de Kroon JFEM, van Bergen CAM, de Paus RA, et al. Human cytotoxic CD8+ T-lymphocyte clones engraft in severe combined immunodeficient (SCID) mice but show diminished function. *J Immunother* 1997;20:101-10.
- Buchsbaum RJ, Fabry JA, Lieberman J. EBV-specific cytotoxic T lymphocytes protect against human EBV-associated lymphoma in SCID mice. *Immunol Lett* 1996;52:145-52.
- Gidlof C, Dohlsten M, Lando P, et al. A superantigen-antibody fusion protein for T cell immunotherapy of human B lineage malignancies. *Blood* 1997;89:2089-97.
- Ghetie MA, Podar EM, Gordon BE, et al. Combination immunotoxin treatment and chemotherapy in SCID mice with advanced, disseminated Daudi lymphoma. *Int J Cancer* 1996;68:93-6.
- Klein E, Klein G, Nadkarni JS, et al. Surface IgM-kappa specificity on a Burkitt lymphoma cell *in vivo* and in derived culture lines. *Cancer Res* 1968;28:1300-10.
- Storkus WJ, Alexander J, Payne JA, et al. The alpha 1/alpha 2 domains of class I HLA molecules confer resistance to natural killing. *J Immunol* 1989;143:3853-7.
- Boon T, van der Bruggen P. Human tumour antigens recognized by T lymphocytes. *J Exp Med* 1996;183:725-9.
- Vonderheide RH, Hahn WC, Schultze JL, et al. The telomerase catalytic subunit is a widely expressed tumour-associated antigen recognized by cytotoxic T lymphocytes. *Immunity* 1999;10:673-9.
- Riethmuller G, Schneider-Gadicke E, Johnson JP. Monoclonal antibodies in cancer therapy. *Curr Opin Immunol* 1993;5:732-9.
- Green MC, Murray JL, Hortobagyi GN. Monoclonal antibody therapy for solid tumours. *Cancer Treat Rev* 2000;26:269-86.
- Shan D, Ledbetter JA, Press OW. Apoptosis of malignant human B cells by ligation of CD20 with monoclonal antibodies. *Blood* 1998;91:1644-52.
- Mathas S, Rickers A, Bommert K, et al. Anti-CD20 and B cell receptor mediated apoptosis: evidence for shared intracellular signalling pathways. *Cancer Res* 2000;60:7170-6.
- McLaughlin P, Grillo-Lopez AJ, Link BK, et al. Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: half of patients respond to a four-dose treatment program. *J Clin Oncol* 1998;16:2825-33.
- Zavazava N, Kronke M. Soluble HLA class I molecules induce apoptosis in alloreactive cytotoxic T lymphocytes. *Nat Med* 1996;2:1005-10.
- Hansen B, Janssen E, Machleidt T, et al. Purified truncated recombinant HLA-B7 molecules abrogate cell function in alloreactive cytotoxic T lymphocytes by apoptosis induction. *Transplantation* 1998;66:1818-22.
- Lone YC, Motta I, Mottez E, et al. *In vitro* induction of specific cytotoxic T lymphocytes using recombinant single-chain MHC class I/peptide complexes. *J Immunother* 1998;21:283-94.
- Weiden PL, Breitz HB, Press O, et al. Pretargeted radioimmunotherapy (PRIT) for treatment of non-Hodgkin's lymphoma (NHL): initial phase I/II study results. *Cancer Biother Radiopharm* 2000;15:15-29.
- Paganelli G, Chinol M, Maggiolo M, et al. The 3-step pretargeting approach reduces the human anti-mouse antibody response in patients submitted to radioimmunoscintigraphy and radioimmunotherapy. *Eur J Nucl Med* 1997;24:350-1.



32. Chinol M, Casalini P, Maggiolo M, et al. Biochemical modifications of avidin improve pharmacokinetics and biodistribution and reduce immunogenicity. *Br J Cancer* 1998;78:189-97.
33. Neri D, Natali PG, Petrucci H, et al. Recombinant anti-human melanoma antibodies are versatile molecules. *J Invest Dermatol* 1996;107:164-70.
34. Tan LC, Gudgeon N, Annels NE, et al. A re-evaluation of the frequency of CD8<sup>+</sup> T cells specific for EBV in healthy virus carriers. *J Immunol* 1999;162:1827-35.
35. Callan MF, Tan L, Annels N, et al. Direct visualization of antigen-specific CD8<sup>+</sup> T cells during the primary immune response to Epstein-Barr virus in vivo. *J Exp Med* 1998;187:1395-402.
36. Khanna R, Bell S, Sherriatt M, et al. Activation and adoptive transfer of Epstein-Barr virus-specific cytotoxic T cells in solid organ transplant patients with posttransplant lymphoproliferative disease. *Proc Natl Acad Sci USA* 1996;96:10391-6.
37. Savoldo B, Heslop HE, Rooney CM. The use of cytotoxic T cells for the prevention and treatment of Epstein-Barr virus induced lymphoma in transplant recipients. *Leuk Lymphoma* 2000;39:455-64.

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